Applicant

Targeted vectors for gene therapy

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ABSTRACT **Successful gene therapy requires not only the identification of an appropriate therapeutic gene for treatment of the disease, but also a delivery system by which that gene can be delivered to the desired cell type both efficiently and accurately. Reductions in accuracy will inevitably also reduce efficiency since fewer particles will be available for delivery to the correct cells if many are sequestered into nontarget cells. In addition, the therapy will have net benefit to the patient only if gene delivery is sufficiently restricted such that normal cells are left unaffected by any detrimental affects of bystander cell transduction. Here we review how currently available delivery systems, both plasmid and viral, can be manipulated to improve their targeting to specific cell types. Currently, targeting is achieved by engineering of the surface components of viruses and liposomes to achieve discrimination at the level of target cell recognition and/or by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain target cell types. In addition, we discuss emerging vectors and suggest how gene therapy delivery systems of the future will be composites of the best features of diverse vectors already in use.— Miller, N., Vile, R. Targeted vectors for gene therapy. *FASEB J.* 9, 190-199 (1995)**

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THE IDENTIFICATION OF THE UNDERLYING genetic defects has recently made gene therapy an attractive treatment option for a wide variety of diseases. However, there is a corresponding requirement to produce vector systems that can deliver therapeutic genes to the appropriate target cells either in vivo or ex vivo. These systems must be both *efficient* and *accurate*. The range of different diseases amenable to intervention by gene therapy means, however, that no single delivery system is likely to be universally appropriate. For instance, the requirements of gene therapy for cystic fibrosis are greatly different from those of cancer. In the former case, only a certain proportion of a localized population of cells needs to be targeted with a single corrective gene; by contrast, cancer gene therapy usually involves the targeting of all of a diffusely spread population of cells, with the ultimate aim of killing rather than correcting them. Hence, the stringency with which the therapeutic gene needs to be accurately delivered can vary greatly. Expression of a copy of the cystic fibrosis transporter gene in nontarget cells is likely to be much less toxic than inadvertant expression of cytotoxic genes, aimed at cancer cells, but expressed in normal bystander cells.

Here, we review the progress in targeting gene delivery systems to specific target cell populations and look forward to the areas of research that will bring developments for the future. Unfortunately, improvements in the accuracy of a vector often compromise its efficiency, and vice versa. Nonetheless, it is clear that the technology now exists to incorporate specific targeting features into most of the currently available delivery systems. These may be at the level of *1)* target cell surface recognition, by manipulating the surface recognition components of viruses and liposomes; or *2)* target cell transcriptional restrictions, by incorporating transcriptional elements into plasmid or viral genomes such that the therapeutic gene is expressed only in certain target cell types.

The ultimate aim for the vectors of the future is to include these and other targeting opportunities within the same vehicle. In all probability, this will involve the incorporation of the most beneficial features of a variety of viral and nonviral systems into a single hybrid vector specifically custom built for each individual therapeutic situation.

TARGETING OF GENE THERAPY VECTORS AT THE LEVEL OF THE CELL SURFACE

Retroviral vectors

A primary determinant of retrovirus infectivity is the interaction between specific receptors on the host cell surface and glycoproteins (Env) on the lipid envelope of the retroviral particle. Ideally, targeted retroviral vectors for human gene therapy would use safe recombinant genomes and packaging lines from wild-type retroviruses that naturally display envelope proteins with the required tropisms. However, few naturally occurring retroviral infections are strictly limited to one cell type (1), and of the known receptors for retroviruses, only the HIV-1/SIV receptor CD-4 (2) is of relatively restricted distribution. Attempts have been made to produce vectors and packaging lines from HIV (3). However, HIV is a complex retrovirus that requires a number of self-encoded autoregulatory proteins, and this complicates the construction of stable packaging lines. Nevertheless, the principle of a recombinant HIV genome as a gene vector for $CD4^+$ cells has been demonstrated (3). However, vectors carrying HIV-1 *env* sequences would have to be used with extreme caution as the HIV-Env protein itself may be neurotoxic (4) or even immunosuppressive.

Most recombinant retroviral vectors and packaging lines produced so far have been based on murine leukemia viruses (MLVs)[2] (5). There are five recognized MLV groups (1) as

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[2]Abbreviations: MLVs, murine leukemia viruses; MLV-E, ecotropic strain of MLV; MLV-A, amphotropic strain of MLV; RES, reticuloendothelial system; PEG, polyethylene glycol; ReSV, respiratory syncytial virus; ASOR, asialoorosomucoid; LCRs, locus control regions; DT-A, diphtheria toxin A; MVM, mouse minute virus.

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defined by tropism, of which the most useful for gene delivery purposes have been the ecotropic strain (MLV-E), which infects virtually all rodent cells, and the amphotropic strain (MLV-A), which infects practically all mammalian cells. Packaging lines have therefore been created to allow production of retroviral vectors with host ranges that are either ecotropic or amphotropic, respectively (5). It is likely that all retroviral vectors suitable for human gene therapy in the near future will be based on such recombinant MLV genomes because they are well characterized with regard to safety and efficiency. For targeted retroviral vectors, then, the problem is either to restrict the promiscuous tropism of amphotropic particles or to confer upon ecotropic particles a limited human cell affinity. This could be done either by: *1)* genetic manipulation of the producer line such that amphoor ecotropic Env is replaced by a different viral or nonviral protein having the required affinity; *2)* directly engineering a particular affinity into Env; or *3)* molecular conjugate approaches, in which ligands are coupled to the outside of the retroviral particle.

Replacement of Env: retroviral pseudotypes

The facility (5) with which *trans*- and *cis*-acting functions can be separated in MLV packaging lines allows easy experimental manipulation of the *trans*-acting function responsible for cellular tropism, namely, Env. This raises the possibility of replacing one viral *env* with that of another, thereby creating a hybrid producer line that generates "pseudotyped" viral vectors with a tropism conferred by the replacement *env* (Fig. 1). Phenotypic mixing has been used for many years as a tool to study receptor interactions (see ref 1 for a review); however, efforts have recently been directed at precisely replacing *env* and producing not envelope mixtures but vector populations exclusively displaying a novel tropism (1, 6). Such hybrid formation in general seems to occur more efficiently between closely related viruses. For instance, a recombinant MoMLV genome can be rescued by C-type viruses but not by HTLV-I r D-type viruses (7). However, provision of h m logous or more closely related Gag proteins in some cases relaxes phenotypic restrictions on efficient pseudotyping of vector genomes with exogenous Env; for instance, an MoMLV vector can be packaged inside HTLV-1 (8) envelopes when MoMLV *gag-pol* are supplied in *trans*. Similarly, HIV has been given an extended host cell range by pseudotyping with the unrelated viruses HSV and VSV (9). Although these examples demonstrate the principle of creating an improved retroviral vector for human gene therapy by pseudotyping, so far they have produced only vectors with extended tropism rather than with restricted specificities.

The logical and necessary extension of pseudotyping approaches, then is to replace retroviral envelope genes with genes derived from nonviral sources. Although there are instances of nonviral glycoproteins being preferentially incorporated into retroviral particles, such as Thy-1 (10) and CD4 (11), actual infection of target cells, as opposed to specific binding, via display of such nonviral proteins has not been demonstrated, and is likely to require either fusogenic sequences within the foreign protein itself or coexpression of fusogenic molecules on the viral envelope.

Engineering Env

Genetic manipulations whereby sequences conferring specific binding affinities are engineered into preexisting viral *env* genes represent a promising approach. In MoMLV the sequences that determine receptor specificity seem t be in the most distal of the two variable regions within the amino-terminal portion of the SU Env subunit, and replacement of the variable region of one strain with that of an ther can, for instance, change viral tropism from that of strain

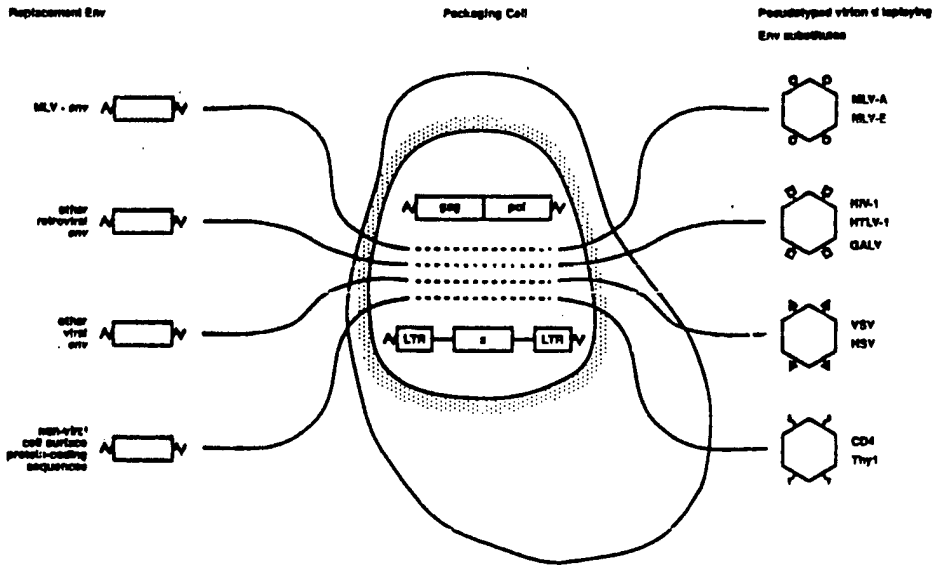

Figure 1. Generation of retroviral vectors with novel tropisms by construction of hybrid packaging lines. Transfection of a cell with genes (*gag-pol, env*) that encode viral *trans*-acting functions allows expression of all the structural components of the virion by that cell; these components can recognize and package the recombinant retroviral genome (shown here bounded by long terminal repeats (LTRs) and carrying a therapeutic gene x). Here we represent diagrammatically the various classes of retroviral pseudotypes that have been produced by providing various *env* genes in *trans*; this illustrates the principle of alteration of retroviral vector tropism by pseudotyping.

4070A to that of 10A1 (12). Engineering of murine retroviral Env proteins is being actively investigated (12–14) and is an important area of research. However, receptor recognition may involve complex interactions between the cellular ligand and different parts of the viral Env, and modification of viral tropism by direct replacement of receptor-binding sequences will not be straightforward. The function of Env proteins is not simply to adhere to host cells but also to participate in a sequence of events leading to membrane fusion. Excessive alteration of Env structure might therefore jeopardize the exposure of hydrophobic domains required for fusion and correct viral internalization. Nevertheless, a mammalian cell tropism has been conferred on an avian retrovirus by engineering integrin-binding sequences into Env. It was found that two of the variable regions of ALV Env could be manipulated by exchanging *env* sequences with those encoding a 16-amino acid RGD-containing peptide to produce Env proteins that were processed and incorporated into retroviral particles (15). Such hybrid envelopes could still efficiently mediate infection of avian cells through the ALV receptor, and could also infect and transfer neomycin resistance to mammalian (ALV-refractory) cells that expressed RGD-recognizing integrins. Infection was not efficient and required previous deglycosylation of the virus to expose RGD epitopes, but it is an important demonstration of the principle of targeting retroviral vectors by envelope modification.

In other studies, the RSV host range has been broadened to include human cells by packaging the genome with a chimeric Env that was a fusion of the RSV signal peptide and the influenza virus hemagglutinin (16). Chimeric Env was found to be incorporated into the virions as efficiently as wild-type RSV Env. It may be possible to use influenza hemagglutinins to direct retroviral vectors to subsets of cells exhibiting particular glycosylation phenotypes as the various influenza strains possess different hemagglutinins with different precise specificities. Another candidate protein for restriction of tropism is the B19 parvovirus surface protein, the surface receptor for which has recently been characterized (17) as the tetrasaccharide of globoside (blood group P antigen), which has a very limited tissue distribution. The B19 surface protein may be susceptible to fine-tuning of saccharide specificity by recombinant techniques or site-directed mutagenesis, similar to the influenza hemagglutinin (18).

The possibility of targeting retroviral vectors to particular glycosylation phenotypes may be of special interest for cancer therapy, as many transformed cells show altered glycosylation. Whether or not any aberrantly expressed glycans can mediate viral entry is another question; a recent report indicates that retroviruses targeted to cells via lectin cross-linking cannot infect the cells after binding (19), but this could be a function of the lectin or of structural alterations caused by cross-linking rather than a function of the glycan receptor.

The demonstrable ability (16) to alter RSV tropism from avian to human cells by manipulation of envelope structure could be of great interest for cancer therapy. This is because the vast number of target cells in malignant disease suggests that either the immune system must be recruited or that a replicating vector be used to target all the tumor cells, and RSV is a replicating vector par excellence. Besides its own genome, this virus is known to carry a cell-derived oncogene; replacement of this with a therapeutic cDNA would give a replication-competent gene therapy vector.

Encouraging results have been reported using a similar approach, in which a cDNA encoding an mAb fragment capable of hapten recognition was fused to the *env* gene of MoMLV (18). Coexpression of this gene with the normal envelope in an ecotropic packaging line resulted in infective viral particles that possessed the appropriate hapten-binding activity. It should be noted that the packaging line was expressing and required parental ecotropic Env as well as the chimeric protein, so it remains to be seen if infective retroviral particles can be assembled that contain only hapten-displaying Env (20). This approach has yet to be demonstrated using a hapten directed against a relevant human antigen capable of mediating virus internalization, and is still far from in vivo application.

Targeting by retrovirus-ligand conjugates

Hepatocytes possess a unique receptor that internalizes asialoglycoproteins. Conjugation of lactose to ecotropic viral particles allowed them to be recognized as asialoglycoproteins and broadened their host range to include human hepatoma cells (21). However, this approach is limited first to cells that express the asialoglycoprotein receptor, and second to proliferating cells (because retroviruses depend on host cell mitosis in order to integrate). As normal liver cells have a very low turnover rate, this technique is most likely to be of use for in vivo delivery to malignant liver disease of the hepatocyte lineage. Furthermore, because the vector was based on an ecotropic virus, its tropism in humans would be limited entirely to hepatocytes, greatly increasing its safety compared with broad affinity vectors such as those bearing the 4070A or GALV envelope proteins.

In a more indirect approach, it was found that ecotropic MoMLV vectors bound to human hepatoma cells after being cross-linked to the transferrin receptor by a series of antibodies; however, there was no subsequent proviral integration, suggesting either that the cross-linking antibodies were inhibiting membrane fusion or that the transferrin receptor cannot mediate appropriate viral internalization (22). A similar cross-linked mAb technique has been used to target ecotropic retroviral particles to human cells in vitro by means of the streptavidin-biotin reaction (23). This allowed ecotropic virus to bind to cells expressing human class I or II MHC antigens and to become internalized and integrated. An extension of this technique (19) showed that biotinylated EGF or insulin could substitute for the anticellular receptor antibody, and that EGF and insulin receptors could mediate internalization, leading to integration, of retroviral particles bearing streptavidin-conjugated antibodies. The possibility of targeting retroviral vectors by means other than murine antibodies, which suffer from numerous disadvantages in vivo, suggests that this approach may have potential although its in vivo applicability has yet to be demonstrated.

Adenoviral vectors

Adenoviruses are double-stranded DNA viruses in which the viral genomic DNA is contained in a virally encoded protein coat (capsid) rather than a phospholipid bilayer of host cell origin. The capsid consists of three major types of subunit: the hexon, which makes up the bulk of the coat; the penton base; and the penton fiber. The fiber is attached to the capsid via the penton base and projects outward; base and fiber together are known as the penton complex. During infection, the fiber mediates initial binding of the virus to an unidentified cellular receptor and the penton base subsequently mediates virus internalization via interactions with α_v-type integrins (24). Thus, the penton complex is respon-

sible for binding and internalization, and therefore for viral tropism at the level of cell recognition. Although adenoviral diseases are usually associated in vivo with respiratory epithelium or the GI tract, their cellular receptors seem to be widely distributed (25). Clearly then, as with retroviruses, the problem is to limit viral tropism to a particular subset of tissues. The adenoviral proteins responsible for attachment and internalization, respectively, have been well characterized, giving two points at which to manipulate tropism. The most promising approach is to restrict adenovirus infection at the cell-binding stage by replacing the carboxyl-terminus knob of the fiber with a ligand conferring a particular tropism, for instance, with an antibody hapten. One report (26) describes the restriction of adenovirus type 5 tropism by a different kind of fiber modification where intact virions were chemically modified so that their fiber carbohydrate groups were covalently linked to an asialoglycoprotein-polylysine conjugate. Such modified virus was found to have much decreased infectivity to asialoglycoprotein receptor-negative cells while retaining infectivity to receptor-positive cells. This approach would be equally applicable to targeting adenoviral vectors per se. It may also be possible to restrict infection by replacing the RGD-containing domain of the penton base with sequences having affinity for a ligand other than RGD-recognizing integrins.

Adenoviral vectors can also be targeted via the route of administration (27); targeting of a *lacZ*-expressing adenoviral vector to the kidney by renal artery or pyelic cavity infusion resulted in β-gal activity in various renal cells with no detectable expression in liver, lung, or bladder cells (27).

A possible advantage of refinement of vector targeting to the point of absolute specificity might be the ability to use replicating vectors for gene therapy. For cancer, development of a replicating adenoviral vector, perhaps carrying a cytokine or suicide gene, targeted to cancer cells at the level of cell binding (via fiber/base manipulations) and at the level of transcription (see next section) might allow transduction of the large number of malignant cells in a tumor deposit; cell death due to adenovirally induced lysis may even potentiate the field effect of cytokines. A safety feature of such a system would be that the immune system would be expected to eventually clear such therapeutic infections (as it does for wild-type infections); therefore this potential therapy only awaits adequate targeting strategies.

Liposome vectors

Most work on targeted liposomes has been designed to deliver cytotoxic drugs to cancer cells and has been reviewed recently (28). Expression of a cDNA in the target cells makes greater demands on the vector system in that it must not only target the appropriate cell type but also allow efficient delivery of undegraded DNA to the nucleus. For most targeted gene delivery purposes, conventional liposomes are limited because of their selective uptake by cells of the reticuloendothelial system (RES), in particular by macrophages resident in liver, spleen, and bone marrow, because of their limited extent of extravasation. Where macrophages themselves are the target, however, RES affinity is advantageous. In *L. donovani* leishmaniasis parasites not only multiply in the Kupffer cells of the liver, but are also resident in a vacuole to which lysosomes fuse, so that liposomes are passively targeted not only to the parasitized cell but also to the appropriate organelle, making liposome-mediated delivery of transcriptionally targeted antisense or suicide genes to these parasites a real possibility. It is also possible in a few cases to avoid much of the RES by the particular route of application, particularly where the target tissue is found in a discrete anatomical compartment; e.g., nontargeted liposomes could be applied directly to the bladder for treatment of carcinoma or to the lung for treatment of cystic fibrosis or αAT deficiency. Targeting by compartment has allowed confined transduction of discrete sections of arterial wall using both liposomal and retroviral vectors (29).

In most cases, however, in vivo use of liposomes requires first avoiding the RES, and second, display of appropriate tropic and fusogenic molecules (Fig. 2). Uptake by the RES can be considerably delayed, but not altogether avoided, by the use of "stealth" liposomes that display negatively charged moieties such as the ganglioside GM1 and polyethylene glycol (PEG) (28). For most systemic purposes, the stealth formula is probably essential.

Liposomes bearing an immunoglobulin complement ("immunoliposomes") can exhibit tropisms conferred by the displayed antibody. Hence, coupling to liposomes of an antibody against glioma cells increased the efficiency of gene delivery to these cells in culture by about sevenfold (30). Just as mAbs may be conjugated to liposomes to confer targeting capability, so may other ligands such as growth factors and hormones. Coupling of transferrin to liposomes followed by i.v. injection in a rabbit model resulted in significantly greater localization to bone marrow erythroblasts (31), and incorporation of surfactant protein A into liposomes increased the uptake of the liposome cargo by alveolar type II cells (32). However, it is not sufficient merely to confer upon the vector a particular binding ability; the particle must bind to a ligand that also allows fusion of liposome and cell membranes. Such considerations of appropriate internalization of vector cargo are especially important for gene delivery vectors, where the DNA must not only reach the appropriate cell type but also must reach the nucleus in undegraded form.

Conjugating virions to liposomes or incorporating viral surface glycoproteins into liposomes might create a vector that has the efficient cell attachment and entry mechanisms of a virus but not the safety drawbacks; much work has been done in this area with Sendai virus in particular (33). Another system used liposomes that displayed only the fusogenic protein of Sendai virus (F-protein) and not the cell-binding protein (hemagglutinin) (34). However, although

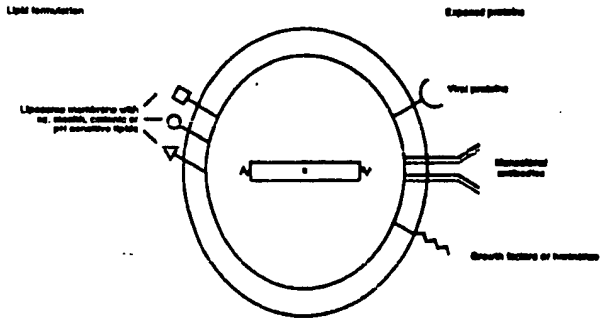

Figure 2. Modification of lipid membranes to produce targeted liposomes. Targeting of liposomes requires first abrogation of their RES affinity, and second, provision with exposed ligands having the required targeting capacity. Inclusion of ganglioside glycolipids into the lipid formulation can allow RES evasion; other lipid formulations include cationic lipids to allow promiscuous membrane binding and hence lysosome escape, and pH-sensitive lipids, which allow lysosome escape without the broad affinity conferred by cationic lipids. Various types of ligand can be inserted into the lipid membrane for provision of particular tropisms (see text for details).

such approaches can make lip somes up to 10-fold more efficient than lipofecti n at gene delivery (33), in terms of targeting all it can do is c nfer upon the liposome the tropism f the virus, and there are very few native viral receptors that exhibit a narrow and precise cell type specificity. Nevertheless, a promising system (35) is currently being developed in which respiratory epithelium is targeted by means of the surface proteins of respiratory syncytial virus (ReSV), which is responsible for infections of the lower respiratory tract. Liposome-type envelopes were constructed that displayed both the attachment and fusion proteins of ReSV, and these have been shown to enter all cells of a cultured respiratory epithelial cell line within 1 h (35).

Cationic liposomes such as the commercially produced lipofectin can efficiently avoid the lysosomal pathway because the particular lipid composition allows direct fusion of liposome and cell membranes. These particles are therefore much more efficient than conventional liposomes, and for in vitro transduction have largely replaced them. Cationic liposomes have also been used for in vivo approaches and even clinical trials; however, there seem to be no data on the extent to which these liposomes can avoid the RES, and indeed the cationic surface would seem to be incompatible with the negative charges characteristic of the stealth formulation. One report suggests that the cationic liposome has as much affinity for other cell types as for the RES after i.v. injection (36). Administration of liposomes carrying SV40-CAT resulted in widespread expression of the marker gene for up to 9 wk, albeit mainly in tissues generally associated with the RES such as spleen, liver, lymph nodes, and bone marrow as well as in vascular endothelium. CAT expression was also observed in tumor cells in this experiment, probably as a consequence of leaky tumor vasculature It may eventually be possible to combine the efficient lysosomal avoidance of cationic liposomes with a specific targeting capacity, although the problem is likely to be that the generally fusogenic nature of cationic liposomes may preclude any precisely restricted targeting.

Molecular conjugate vectors

Targeting of plasmid DNA may be achieved by coupling the DNA to a ligand with a demonstrated cell or tissue affinity. This is usually brought about by covalently linking a polycation such as polylysine to the ligand; the polycation can then bind to and condense plasmid DNA via electrostatic interactions, leaving the ligand exposed on the surface of the conjugate (37). The ligands chosen must be efficiently endocytosed in the target cells so that DNA is efficiently internalized. One of the first receptors to be used in this way was the asialoglycoprotein receptor, whose expression is limited to hepatocytes; this receptor binds glycoproteins with terminal galactose residues for removal from the circulati n; asialoorosomucoid (ASOR) is a major natural ligand for this receptor. BSA has been given specificity for the ASOR receptor by artificial galactosylation, and has been used to target CAT and human factor IX cDNAs (38) to hepatoma cells in vitro and to liver but not other tissues in vivo. Other ligands that have been used in similar conjugates include insulin (39), EGF (40), lectins (41), and transferrin (37). A major drawback of classical molecular conjugate vectors is that internalization depends on receptor-mediated endocytosis, a process that directs the receptor complex to lysos mes where it is degraded; only a small fraction of introduced

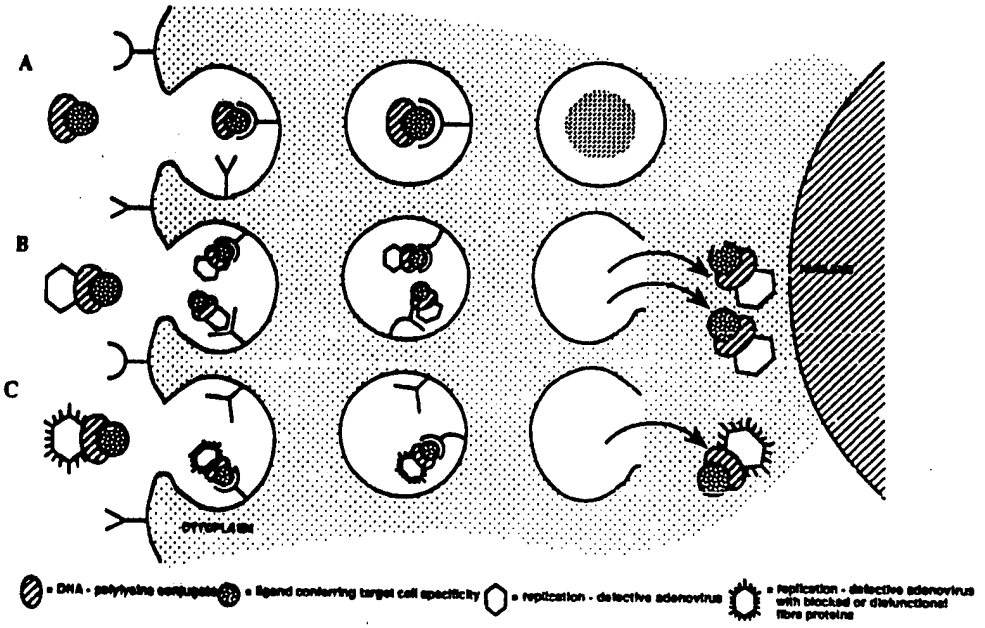

Figure 3. Targeting of plasmid DNA by molecular conjugate vectors. Conjugation of plasmid DNA to a particular ligand can confer a particular targeting capacity, but results in a vector of very low efficiency because most receptor-mediated endocytosis directs such conjugates to lysosomes where the great majority of vector DNA is degraded (route A). By complexing an adenovirus coat to the conjugate, a highly efficient vector is created by virtue of the ability of adenovirus proteins to disrupt the endosome before vector degradation (route B); however, this abrogates any targeting capacity conferred by the ligand, as the complex can enter cells either via the ligand receptor or via the virtually ubiquitous adenoviral receptor. To truly target such complexes it will be necessary to use modified adenoviral coats that retain the lysosomal escape mechanism but cannot interact with the adenoviral receptor (route C).

DNA escapes this pathway and enters the nucleus, leading to low efficiency of transduction.

A new generation of molecular conjugate vectors has been produced that has the capacity to escape the degradative lysosomal pathway by utilizing features of the adenovirus capsid (Fig. 3). Adenovirus disrupts endosomes during cell entry as a consequence of a conformational change in the capsid proteins, resulting in membrane breakdown, triggered by a drop in pH. Hence, molecular conjugate vectors delivered DNA to cells with greatly increased efficiency when transfection was done in the presence of adenovirus. However, this effect relies on both virus and vector being present in the same endosome. To improve efficiency, the adenovirus has been coupled directly to the molecular conjugate (37). However, adenovirus receptors are virtually ubiquitous and so the coupling of an adenovirus receptor to a targeted molecular conjugate would be expected to partially or completely abrogate any preferential tropism conferred by the ligand. Blocking the interaction of fiber with adenovirus receptor by mAb to the fiber resulted (42) in a vector that was both targeted to a specific subset of cells and able to escape the lysosomal pathway. A more satisfactory approach would be to create recombinant adenoviral vectors that display dysfunctional fiber proteins in order to bypass the antibody-coating step.

Few in vivo experiments have been attempted using adenovirus-molecular conjugate complexes, and in fact it is unlikely that such vectors will be routinely applicable to in vivo work, although they are likely to be of use for ex vivo strategies (43). This is a consequence first of the size of the complex (transferrin-polycation conjugates are approximately 100 nm in diameter (44); complexed with AdV they would be even larger), which will prohibit extensive extravasation or tissue penetration, and second, of the likelihood of direct immunogenicity of the AdV proteins (45).

TARGETING OF GENE THERAPY VECTORS AT THE GENETIC LEVEL

Transcriptional targeting

Therapeutic cDNAs may be limited in expression to a particular subset of cells by placing them under the control of regulatory elements that possess binding sites for tissue-restricted positive or negative *trans*-acting factors (Fig. 4). Correctly regulated expression may require, in addition to 5' promoter sequences, distant elements either 5' or 3' to the coding region; these elements act together with the promoter and allow tissue-specific expression at appropriate levels independent of position of integration. Such locus control regions (LCRs) have been identified for a number of genes. LCRs would be of much use for gene augmentation but the transfer of such large sections of DNA to target cells will be problematic, particularly in vivo, and in fact for the foreseeable future may be confined to ex vivo strategies. Where a monogenic defect results in pathology in more than one tissue, the most pragmatic approach to appropriately limit the expression of therapeutic cDNA is to use the cellular promoter/enhancer elements native to the defective gene. Furthermore, the use of cellular rather than viral promoters reduces the chance of loss of cDNA expression due to inactivation of viral sequences by methylation or other mechanisms (46). Thus, cellular promoters may confer benefits both of long-term expression and of tissue-restricted expression, and where vector-targeting at the cell-binding level has not been achieved it may represent the only way of limiting expression of exogenous cDNA.

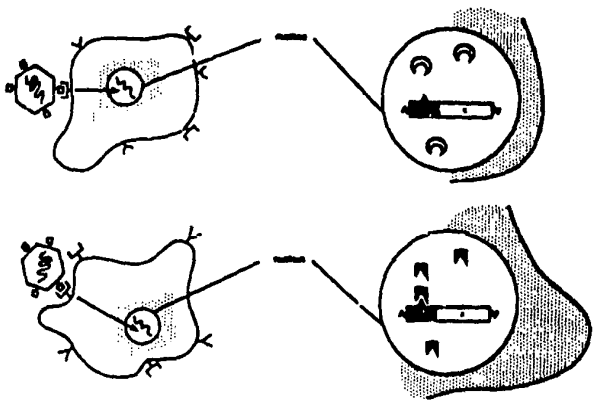

Figure 4. Tissue-restricted transcription. A promiscuously binding vector can be targeted at the transcriptional level if the therapeutic gene (x) is controlled by 5' regulatory elements (shown here as a shaded region upstream of x) active only in the presence of tissue-specific nuclear transcription factors; thus expression of *x* occurs only in the target cells.

Tissue-specific cellular regulatory elements have great potential for development of safe, targeted vectors for gene therapy. For example, the creatine kinase promoter has been used in a plasmid vector to restrict dystrophin cDNA expression to skeletal and cardiac muscle, and in the *mdx* mouse model of Duchenne muscular dystrophy, mice transgenic for this promoter-cDNA construct were found to exhibit correction of dystrophic symptoms (47). A potential approach to the treatment of B cell lymphoma involves expression of suicide genes transcriptionally regulated by promoter/enhancers from the Ig heavy chain or the κ light chain genes; expression plasmids containing the diphtheria toxin A (DT-A) gene controlled by these regulatory elements mediated significant expression of DT-A in B lymphoid cells but not in HeLa cells or fibroblasts (48).

Endothelial cells are attractive recipients for gene transfer therapies not only for obvious purposes such as targeting of tumor vasculature or therapy of cardiovascular disease, but also for the systemic secretion of therapeutic factors. An endothelial cell-specific regulatory region has recently been characterized (49) as 500 bp of 5' sequences, associated with the gene for von Willebrand's factor, acting in conjunction with an essential region in the first intron. This promoter could be particularly useful when driving a suicide gene in a retroviral vector as it would then be targeted to dividing endothelial cells, i.e., almost exclusively tumor vasculature.

Tissue-specific cellular promoters frequently retain their specificity in the context of a retroviral vector (50); however, this is not always the case, and the design of the retroviral vector may have significant effects on tissue specificity due to promoter interference (51). Tissue-specific promoters have also been shown to appropriately restrict cDNA expression in the context of recombinant adenoviruses, e.g., the rat albumin promoter maintained its hepatoma cell specificity in vitro (52), albeit at low levels.

Antiviral therapy using transcriptional targeting

Transcriptional targeting may be of particular use in the therapy of particular kinds of viral infection. In cases where the viral life cycle depends on self-encoded autoregulatory

proteins, vectors can be made in which therapeutic cDNAs are transcriptionally regulated by these same viral proteins. Transcription of the therapeutic cDNA is therefore limited to cells that are infected by the virus, and thus such an approach could be either prophylactic or curative. This strategy has been applied to experimental HIV therapies. One recent report (53) described the construction of a recombinant retrovirus containing HSV-TK driven by the HIV-2 LTR-TAR; cells expressing this construct became susceptible to ganciclovir after infection by HIV-2 in vitro.

Targeting proliferating cells

Murine C-type retroviral vectors can combine the ability to express cDNA from an internal tissue-specific promoter with an innate tropism for proliferating tissue. Therefore, they have great potential as vectors for the gene therapy of cancer, because restricted cDNA expression is of particular importance in strategies that involve delivery of cytokine or suicide genes and malignancies are often distinguished by rapid division in a relatively quiescent background. Indeed, in a very few cases the retroviral requirement for cell division may be sufficient in itself to target the therapy (Fig. 5); where tumors arise in the CNS their high rate of proliferation in the context of a completely postmitotic tissue, in an anatomical compartment that is separated from the rest of the body, allows efficient targeting with retroviral vectors (54). As an additional targeting feature for malignancies of the CNS, the glial-specific promoter region of the mouse myelin basic protein gene has been used to drive HSV-TK in a retroviral vector (55); this approach could allow long-term administration of producer cells at the primary site or systemic vector application to treat metastatic deposits as collateral infection of nonglial cells would not result in expression of the suicide gene.

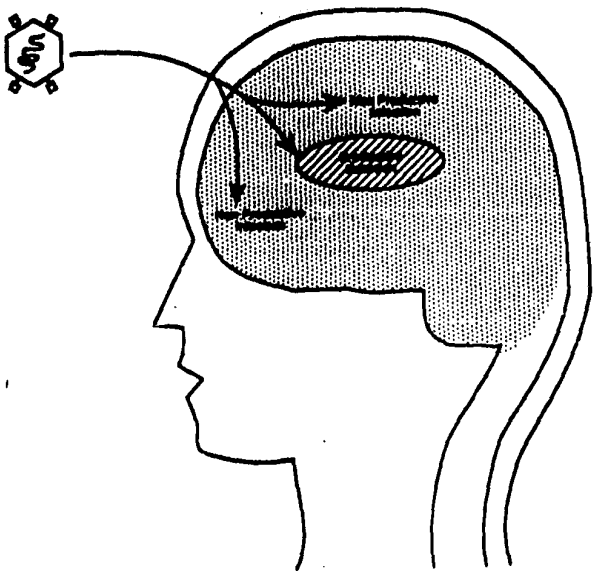

Figure 5. Targeting proliferating cells. Retroviral vectors require cell division for integration and gene expression; therefore where a tumor arises in a completely postmitotic background, such as the CNS, the proliferation of the malignant tissue may be sufficient in itself to allow efficiently targeted delivery of suicide genes via recombinant retroviruses. Actively replicating (tumor) cells are represented by diagonal lines; quiescent neuron tissue is represented by dots.

Retroviral vectors would also be useful in targeting liver malignancies, as the liver is also slowly proliferative under normal circumstances. Tissue-specific promoters would be essential for such strategies, because unlike the CNS, the liver is not efficiently insulated from the rest of the body. Amphotropic retroviral vectors have been constructed carrying HSV-TK cDNA driven either by the albumin or the a-fetoprotein promoters (56). The albumin promoter was active only in cells of the liver lineage; the a-fetoprotein promoter conferred an extra level of targeting in that it was hepatoma-specific as opposed to hepatocyte-specific (a-fetoprotein is normally expressed only in fetal tissues).

The 5' region of the tyrosinase gene has also been used to restrict expression of therapeutic cDNAs to melanocytes and melanoma cells both in vitro and in vivo by means of retroviral vectors (51, 57). This kind of transcriptional targeting may be useful in VDEPT approaches for melanoma because normal melanocytes are dispersed and of low density in body tissues, and their ablation is likely to be minimally pathological. Even better would be the usurpation of tumor-specific transcriptional regulation by using promoter sequences from genes whose overexpression is limited to transformed tissue. One such candidate is the oncogene ERBB2, which is overexpressed in a variety of tumors. The ERBB2 promoter sequences have been used to drive cytosine deaminase cDNA in a retroviral vector (58); this strategy conferred sensitivity to ERBB2-overproducing cells but not to control cells, and represents a potentially widely applicable method of tumor-preferential transcriptional targeting. The a-fetoprotein promoter is in effect completely tumor-specific, but is applicable only to malignancies of the liver.

Exploitation of natural viral tropisms

An obvious approach to the precise targeting of tissues is to make vectors from viruses that have preferential patterns of transcription in target tissues, such as HSV vectors for nervous tissue. However, careful dissection of the genomes of these viruses will be necessary to separate pathogenic sequences from those that confer transcriptional specificity; in most cases it will be preferable to use cellular promoters in the vector of choice, especially as the range of transcriptionally targeted viral genomes is not great.

There may be one remarkable exception to the general requirement for cellular promoters rather than viral promoters in gene therapy, namely, the use of autonomous parvoviral sequences for targeting transformed cells (see ref 59 for review). These viruses preferentially kill transformed cells (60), and coinjection of mouse minute virus (MVM) and Ehrlich ascites tumor cells into the peritoneal cavities of mice inhibited tumor formation by up to 90%. Furthermore, mice that had survived one such coinjection were resistant to a second tumor challenge 5–6 wk later. The precise basis of parvovirus oncotropism is not understood but may be related to an effect of the transformed cell environment on the production or activity of parvovirus autoregulatory proteins. The parvovirus promoter that is preferentially transactivated in certain transformed cells is clearly a candidate to control transcription of suicide or cytokine genes in parvovirus vectors for cancer therapies. Recombinant parvovirus vectors have been made and shown to both transfer exogenous cDNA expression to recipient cells and retain their oncotropism in vitro (61) for human and murine cells. Recombinant parvoviruses may therefore represent one of the most promising approaches to cancer therapies for the future.

Targeted integration: site-specific recombination

Nonintegrating vectors are adequate for transient expression of cDNA. Where the object is a "one-shot" treatment for cure of a genetic disorder, it is necessary to use either an integrating vector or a stably replicating extrachromosomal element. For the future, sequences containing mammalian origins of replication or even entire mammalian artificial chromosomes (62) could have great potential especially for ex vivo approaches. Similarly, vectors based on the Epstein-Barr virus, which is stably maintained episomally as a plasmid in human cells, may one day be suitable for clinical use.

The ideal approach would be to target the exogenous DNA to the mutant gene, i.e., gene replacement rather than gene augmentation. Such gene targeting approaches may be of use for ex vivo strategies to stably transduce cells with less likelihood of simultaneous transformation (63). Such in vitro homologous recombination may be useful in inactivating genes responsible for MHC class I expression in myoblasts to create a universal carrier cell that can be transplanted regardless of the recipient HLA type (63). This approach is applicable to any ex vivo strategy that requires implantation of viable transduced but otherwise unchanged cells. The technology required to accomplish this at levels of efficiency relevant to in vivo gene transfer does not yet exist and so integrating gene therapy vectors at present can offer only gene augmentation.

Nontargeted integration could be hazardous if completely random, not only by turning on downstream oncogenes via promoter readthrough but also by direct disruption of genes, and this is the main source of concern with regard to the use of retroviral vectors in humans. Vectors with the capacity for site-specific integration would overcome these problems. Adeno-associated virus is a defective parvovirus that potentially is widely applicable in gene transfer strategies because it is tropic for many cell types, nonpathogenic in humans (in the absence of helper virus the AAV genome does not replicate but integrates into the genome and assumes a state of latency), and can be manipulated to derive recombinant genomes capable of vectoring exogenous DNA (64). Although these vectors can package only up to 4.5 kb as compared with the retrovirus limit of approximately 7 kb, they are said to have one major advantage over other integrating vectors, namely, a propensity (which is far from total) for apparently harmless integration into a region of human chromosome 19 known as AAVS1 (see review, ref 65). Where such specific integration occurs, it is almost certainly mediated by virally encoded proteins with affinity both for the target site and for the virus genome (66). Although integrated viral sequences remain dormant until superinfection by AdV/HSV, exogenous cDNAs driven by internal promoters can still be active (furthermore, the transcriptional inactivity of the viral ITR means that there will be no promoter interference leading to, for example, loss of tissue specificity of exogenous promoter, and less chance of insertional mutagenesis for the same reason). Thus AAV vectors have been shown to confer neomycin resistance and in some cases to integrate with site specificity (64). This study also showed that AAV vectors preserved their site specificity after transfection in plasmid form; the use of a transfectable plasmid rather than a viral vector might overcome the packaging limitations of AAV vectors (64). It must be said, however, that some groups report that recombinant AAV vectors show site specificity in only a relatively minor proportion of the total number of integration events. There have been several attempts to explore the therapeutic potential of AAV vectors, e.g., the delivery of cDNA for the correction of the cystic fibrosis defects (67).

There may be other vector systems also capable of site-specific integration. Eukaryotic genomes harbor large numbers of endogenous transposable elements of various types (68), i.e., autonomously replicating units that can insert themselves into the host genome. Some of these elements, known as LTR retrotransposons, are very similar to retroviruses both in replication cycle and in organization, being bound by LTRs and possessing coding regions with homology to retroviral *gag-pol* genes. The replicative cycle of LTR retrotransposons exactly parallels that of the retroviruses except that there is no envelope stage, thus, cytoplasmic virus-like particles (69) are formed containing reverse transcriptase, the RNA form of the retrotransposon, and cellular tRNA primers for reverse transcription. Such elements include *copia*, yeast Ty, and the intracisternal A particle of mice; clearly they have great potential as vectors of improved safety as their use with retroviral packaging lines would be less likely to result in helper virus production through homologous recombination. Indeed a mouse retrotransposon VL30 has already been made into a gene transfer vector (70), which can be produced in a standard retroviral packaging line. Endogenous retrotransposons a priori would be expected, through coevolution with the host genome, to display a degree of site specificity of integration as continuous random retrotransposition would be deleterious to the cell. Yeast retrotransposons offer the best examples of site-specific retrotransposons, and moreover, their site of integration appears to be benign. Two of the five *Saccharomyces cerevisiae* retrotransposons, Ty1 and Ty3, exhibit unambiguous site specificity of integration (71). Ty3 elements integrate into sites upstream of genes transcribed by RNA pol III, frequently within 1–4 nucleotides of the start site of transcription. It has been suggested that this sequence-independent site specificity is brought about by interaction of the retrotransposon with elements involved in RNA pol III-mediated transcription, e.g., TFIIIB (71). Similarly, Ty1 preferentially integrates upstream of tRNA genes (71) 57% of insertions occurring within 400 bp of a tRNA gene. A consequence of this specificity is that yeast genes are only rarely interrupted by Ty1 insertions as regions upstream of yeast tRNA genes rarely contain open reading frames (71). The great similarity of LTR retrotransposons to retroviruses allows them to be made into vectors with conventional retrovirus packaging lines (70); possibly the development of a packaging line that provides retrotransposon rather than retroviral *gag-pol* in *trans* will allow the production of vectors with integrational site specificity.

SUMMARY AND PERSPECTIVES

Of the gene therapy protocols that have so far entered clinical trials, targeting of the appropriate vectors has been achieved largely only by indirect means. Thus, several such trials (for example, for treatment of ADA deficiency, HIV infection, or cancer) have used specific cell populations that have been removed from the patient and infected in vitro by nontargeted amphotropic retroviruses before being returned in vivo. Further levels of targeting have been achieved in some cases by careful choice of the patient's cells; for instance, ex vivo transduction of tumor infiltrating lymphocytes with potentially tumoricidal genes has been proposed as a means of delivering their products to tumor deposits at much higher concentrations than would otherwise be possible.

In contrast to ex vivo manipulation of target cells where the vector requires very little, if any, intrinsic targeting capability, there are an increasing number of protocols in which

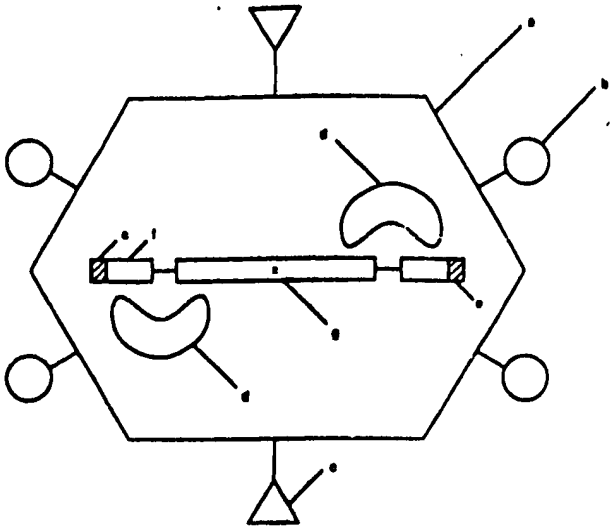

Figure 6. A theoretical composite vector. Some features that might be incorporated in an ideal synthetic vector include a stable, nonimmunogenic envelope, probably lipid (*a*); exposed ligands to confer a particular affinity on the vector (*b*); moieties that encourage fusion between vector and target cell membranes (*c*); proteins to allow directed integration of vector DNA, e.g., site-specific recombinases (*d*); sequences to enable homologous recombination between vector DNA and particular loci of the target genome (*e*); tissue-specific promoter regions to allow restricted expression of the therapeutic gene (*f*); and the therapeutic cDNA (*g*).

recombinant genes are delivered directly to patients in vivo (such as for the treatment of cystic fibrosis and cancer). Once again, targeting at the level of the vector has not yet been particularly well developed; hence, liposome or viral-mediated delivery of the CFTR gene to airway epithelial cells of CF patients has relied largely on the localized delivery of the vectors directly to the affected tissues, and on the fact that there is good evidence that inadvertant expression of the CFTR gene in cells other than the target epithelial cells may have few adverse effects. Localized delivery has also been used in the treatment of brain tumor deposits, using stereotactic injection of retroviral producer cells, but with the added sophistication that the retroviruses would be expected to infect only the actively dividing tumor cells and not the surrounding neural tissue.

However, for the long-term success as well as the widespread applicability of human gene therapy, there will have to be advances in the ability with which clinicians can confidently administer recombinant vectors for the treatment of genetic disease directly to affected tissues in vivo. For this to occur, many targeting strategies outlined in this review, which are currently only at the experimental level, will have to be translated into components of safe and highly efficient delivery systems. Vectors have already been developed that incorporate transcriptional specificity for a certain tissue type; however, the development of surface targeting has been more problematic in most cases. The biggest challenge for the next 5 years will be to combine targeting with efficiency in the production of the vector systems of the future. So far, attainment of one usually compromises the other; for example, we have constructed retroviral vectors targeted at the level of transcription to melanoma cells but these viruses are generally of lower titer than their nontargeted counterparts.

Nonetheless, the imagination and the technology is currently available to allow us to hope that vectors will eventually be constructed that can include both efficiency and specificity. In particular, it does not seem unrealistic to suppose that the gene therapy vectors of the future will not be based exclusively on any single virus or physical vector system alone but will be synthetic, custom-designed vehicles (Fig. 6) into which specific targeting features can be included depending on the particular clinical requirements of the target disease and tissue.

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Prevention of Autoimmune Disease by Retroviral-Mediated Gene Therapy[1]

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T lymphocytes have been implicated in a variety of autoimmune diseases, and therefore one potential therapeutic approach would be to tolerize the pathogenic self-reactive T cells. In this study, we examined whether retroviral gene therapy could be used to induce tolerance and prevent autoimmunity using a transgenic mouse model for experimentally induced diabetes. In this model, the lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp) is expressed on the β-islet cells of the pancreas under the control of the rat insulin promoter (RIP). Previous work showed that the T cells specific for the gp remain unaware of the transgenic gp Ag expressed by the islet cells, and infection with LCMV leads to immune-mediated diabetes. To tolerize the gp-specific pathogenic T cells, a retroviral vector (RV) expressing the LCMV gp was constructed, RV-gp. Replication-defective recombinant retroviruses were used to transduce bone marrow cells, which were subsequently infused into host RIP-gp transgenic animals. Unlike control animals, RV-gp chimeric animals did not possess T cells specific for the gp Ag as measured by proliferation and cytotoxic function, and further analysis suggested that tolerance of the gp-specific self-reactive T cells occurred by clonal deletion. Further experiments demonstrated that chimeric RIP-gp transgenic animals generated using bone marrow transduced with RV-gp did not develop experimentally induced diabetes. Our animal model demonstrates that retroviral gene therapy may cure immune-mediated diabetes by providing long lasting Ag-specific tolerance. *The Journal of Immunology,* **1995, 155: 5404–5408.**

Autoimmune disease is often mediated by T cells that have not received tolerogenic signals to sequestered self Ags (1, 2). Several natural animal models of experimentally induced and spontaneous autoimmunity, as well as transgenic models, have been studied to further understand how these T cells escape self-tolerance mechanisms and become activated to induce immunopathologic destruction (3–10). An ideal approach to treating autoimmune disease would be to manipulate the T cell repertoire of the host so that auto-Ag-specific tolerance can be induced. In the past, retroviral vectors (RVs) have been used to attempt to eliminate superantigen or alloreactive T cells (11, 12). Another possible goal would be to tolerize T cells of defined specificity and eliminate autoimmune disease in a defined model.

Gene therapy has been considered an approach for treating a broad spectrum of genetic disorders and cancer, as well as cardiovascular disease and AIDS (13–15). Delivery of the gene may be accomplished by several techniques, however the majority of studies have employed RVs for gene transfer. Replication-incompetent retroviruses may be generated at high titers that can efficiently transduce a variety of cell types ex vivo, before adoptive transfer in vivo (16–19).

In this study, we examined whether bone marrow cocultured with retroviruses expressing a defined self Ag could generate specific T cell tolerance in reconstituted chimeric animals. Two basic mechanisms of T cell tolerance have been defined, clonal deletion and clonal inactivation (20). Several studies have shown that T cell tolerance may be readily induced against determinants expressed by bone marrow-derived cells (21–23). We therefore attempted to determine whether bone marrow cells that were genetically modified by retroviral gene delivery were sufficient to induce tolerance against a specific autoantigen and ultimately prevent experimentally induced diabetes.

We have previously described a transgenic mouse model, RIP-gp[3] that expresses the lymphocytic choriomeningitis virus gp (LCMV-gp) on the β-islet cells of the pancreas. LCMV-gp-specific T cells in these mice are not tolerant to the LCMV-gp expressed on the pancreas, and on activation by infection with LCMV, the gp-specific T cells infiltrate and destroy the islets, leading to diabetes (5, 24). To tolerize the gp-specific T cells, we generated a RV that expresses LCMV-gp (RV-gp). The following studies were done with chimeric animals that were made using bone marrow transduced with recombinant retroviruses. Experiments addressed whether LCMV-gp-specific T cell tolerance could be induced, and whether immune-mediated diabetes could be prevented in our animal model.

Materials and Methods

Mice

RIP-gp (Bln line) (5) and TCR (327 line) (25) transgenic lines were bred and typed as previously described (24), in specific pathogen-free conditions according to institutional guidelines. RIP-gp animals were generated in the

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[3] Abbreviations used in this paper: RIP; rat insulin promoter, gp; glycoprotein, LCMV; lymphocytic choriomeningitis virus, RV; retroviral vector.

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C57Bl/6J strain, while the TCR line has been backcrossed six times with C57Bl/6J mice. Donor C57Bl/6J animals were purchased from The Jackson Laboratory (Bar Harbor, ME).

Retroviral vectors

RV-gp was generated by cloning the 1.6-kb *Bam*HI LCMV-gp cDNA into the *Bgl*II site of the MSCV RV described previously (19). This vector includes variant long terminal repeats and a mutated 5' untranslated region for efficient expression in hematopoietic cells. The ψ^+ region includes sequences necessary to generate high viral titer of replication defective retroviruses. The original MSCV vector was used as a control, RV-neo.

Northern blot analysis

RNA was extracted from cell lines using the guanidine isothiocyanate method (26). Ten micrograms of total RNA were run on a 1% formaldehyde gel and transferred to a nitrocellulose filter. The positive control sample contained 0.5 μg of RNA from LCMV-infected cells with 10 μg of carrier RNA from uninfected National Institutes of Health (NIH) 3T3 cells. Filters were prehybridized in 50% deionized formamide, 5X SSPE, 0.5% SDS, 5X Denhardt's solution, and 1 mg/ml salmon sperm DNA for 4 h. ^{32}P-labeled LCMV-gp specific probe was generated (Multiprime labeling kit; Amersham, Bucks, UK), and 1 × 10^6 cpm/ml was added to the prehybridization mix for 20 h at 42°C. Filters were washed and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 18 h.

Generation of chimeric animals

Eight-week-old female C57Bl/6J or TCR transgenic mice were used as bone marrow donors. Mice were injected with 150 mg/kg 5-fluorouracil and 4 days later, the bone marrow was harvested. After erythrocyte lysis in 0.17 M ammonium chloride, cells were cultured at 5 × 10^5 cells/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 50 μM 2-ME, 10% heat-inactivated FCS (HyClone, Logan, Utah), supplemented with IL-3 and IL-6 conditioned media as previously described (27). After 48 h, bone marrow cells were cocultivated with a subconfluent monolayer of fibroblasts producing appropriate replication defective retroviral particles in the same conditioned media, which included 8 μg/ml polybrene. After a further 48 h, nonadherent bone marrow cells were harvested, centrifuged, and resuspended in the presence of 0.75 mg/ml G418 (Life Technologies, Inc., Grand Island, NY). Cells were harvested after 24 h and 5 × 10^5 to 2 × 10^6 cells were infused into irradiated recipients (900 rads) by i.v. tail vein administration. Host animals were reconstituted for 10 wk to 5 mo before further experiments were done.

Cytotoxic assays

MC57G fibroblast target cells (H-2^b) were either infected with LCMV or recombinant vaccinia viruses expressing the LCMV-gp or LCMV-np and labeled with ^{51}chromium for 2 h. Target cells (1 × 10^4) were incubated with spleen effector T cells from C57Bl/6 mice infected in vivo 8 days before the assay, at various ratios in a final volume of 200 μl IMDM and 10% FCS. The cells were incubated in 96-well round-bottom plates for 4 to 5 h at 37°C, and 70 μl of the supernatant was removed and counted. Percent specific release was calculated as (cpm experimental release − spontaneous release)/(total release − spontaneous release) × 100.

Proliferation assay

Single cell suspensions were made from the spleen, and 1 × 10^5 cells were cocultured with 2 × 10^4 stimulator cells in 96-well flat-bottom plates. The peritoneal macrophages from LCMV-infected or uninfected mice that were used as stimulator cells were obtained from mice given an i.p. injection of 2 ml thioglycollate 6 days before the assay. Cells were incubated for 48 h in IMDM plus 10% FCS, 2 × 10^{-5} M 2-ME, glutamine and penicillin, and streptomycin (100 U/ml), and then 1 μCi of [^{3}H]thymidine (NEN, Boston, MA) was added to each well. After 16 h, the cells were harvested and counted on a Matrix 96 direct β-counter (Canberra Packard, Meriden, CT).

Induction of diabetes

Mice were given an i.v. infusion of 2000 plaque-forming units LCMV (Armstrong). Blood glucose levels were monitored regularly, and quantitated using a Reflolux III (Boehringer Mannheim, Laval, QC).

Detection of retroviral DNA in chimeric animals

Tissues from various organs were homogenized in PBS and incubated with 0.2 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C. Samples were extracted with phenol chloroform several times and then precipitated. DNA (0.5 μg) was amplified using neomycin-specific primers (CCGGTCCCCTGAATGAACTGC) (CAATATCACGGGTAGC CAACG) for 30 cycles. Ten microliters were electrophoresed on a 1% agarose gel and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). A neomycin-specific probe (3 × 10^5 cpm/ml), generated by random priming (Amersham Multiprime labeling kit), was added to a hybridization solution (5X SSC, 5X Denhardt's solution, 0.1% SDS, and 10% dextran sulfate) and incubated overnight at 65°C. After two washes in 2X SSC, 0.1% SDS, and 0.2X SSC 0.1% SDS, autoradiographs were developed after a 2-h exposure.

Results

To determine whether retroviral gene therapy could be used to alter the T cell repertoire and tolerize pathogenic T cells, a RV was constructed to express the model autoantigen LCMV-gp. The LCMV-gp cDNA was cloned in the *Bgl*II site of MSCV (RV-gp) (Fig. 1*A*). Vector DNA was transfected into a packaging cell line that provides the necessary proteins in *trans* for production of recombinant RV-gp as infectious replication-defective viral particles (28). Cell lines were selected that were capable of exporting recombinant RV-gp retrovirus at high titers (10^6 CFU/ml). Control cell lines were also established that produce a high titer of the MSCV vector (RV-neo).

To determine whether the recombinant retrovirus expressed LCMV-gp, NIH 3T3 cells were infected with RV-gp or RV-neo, and G418-resistant cell lines were established. Northern blot analysis, using a probe specific for LCMV-gp, indicated that LCMV-gp mRNA was present only in cell lines infected with LCMV or RV-gp (Fig. 1, *B* and *C*).

We examined whether RV-gp could induce tolerance of LCMV-gp-specific T cells by several criteria. Assays were done to determine whether functional LCMV-gp-specific CTL were detectable in chimeric animals. RV-gp or RV-neo transduced bone marrow was used to reconstitute irradiated RIP-gp mice (27, 29). These mice and control C57BL/6 mice were infected with LCMV and 8 days later, the spleen cells were assayed for cytotoxic function (Fig. 2). LCMV-gp-specific CTLs were not detected in chimeric mice receiving bone marrow transduced with RV-gp, whereas an efficient LCMV-gp-specific cytotoxic response was seen in control animals. Effector cells from all mice lysed target cells infected with LCMV or target cells expressing LCMV nucleoprotein (np), but did not lyse fibroblasts infected with the control vaccinia virus. This assay shows that a functional LCMV cytotoxic response is generated in chimeric animals treated with RV-gp due to the recognition of other viral epitopes such as the np. The fact that a cytotoxic response against the LCMV-gp was absent, demonstrates that T cell tolerance is highly specific and does not compromise the host repertoire against other foreign determinants.

To examine the mechanism of tolerance, we used TCR transgenic mice expressing the Vα2/Vβ8.1 TCR specific for the LCMV-gp presented in the context of H-2D^b (25). Bone marrow from TCR transgenic mice was transduced with either RV-gp or RV-neo and infused into irradiated C57BL/6 mice. The thymus and lymph nodes were analyzed 10–12 wk later for the presence of the transgenic TCR using mAbs specific for the Vα or Vβ-chains. Mature CD8$^+$ thymocytes from RV-neo control animals expressed the transgenic TCR, whereas CD8$^+$ thymocytes from RV-gp animals did not show any detectable T cells expressing the transgenic αβ heterodimer (data not shown). Figure 3*A* indicates that Vα2$^+$ CD8$^+$ and Vβ8$^+$ CD8$^+$ lymph node T cells were present in chimeric mice that were reconstituted with RV-neo transduced bone marrow, whereas less than 1% of peripheral T cells were detected in mice that were reconstituted with RV-gp transduced bone marrow. However, in mice reconstituted with RV-gp-treated bone marrow, a significant population of Vα2 and/or Vβ8.1 T cells was present that did not express the CD8 coreceptor. This demonstrates that the TCR transgenic bone marrow partially reconstituted the

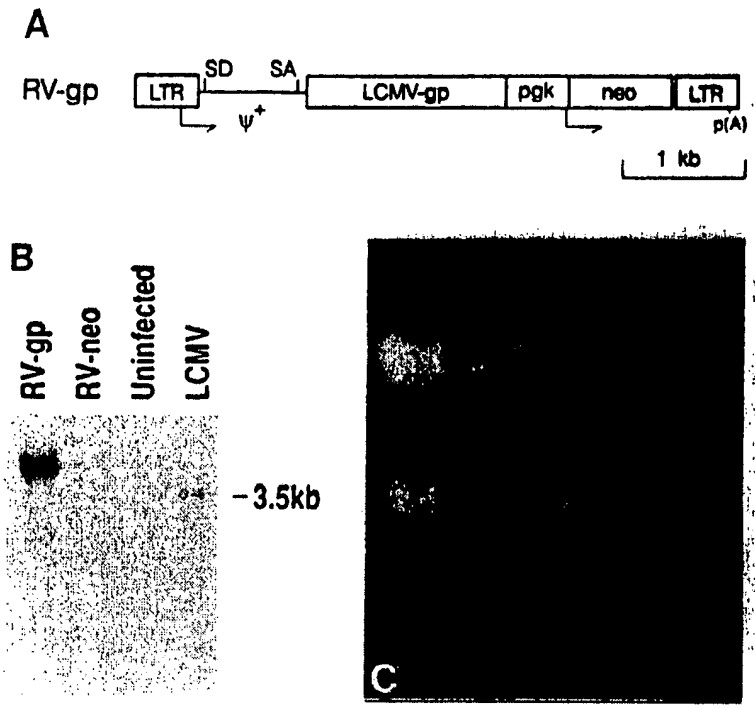

FIGURE 1. Expression of LCMV-gp in fibroblasts transduced with RV-gp. *A*, Structure of RV-gp. Arrows indicate long terminal repeats and phosphoglycerate kinase (pgk) promoters utilized for expression of the LCMV-gp cDNA and the *neo* gene, respectively. SD, splice donor site; SA, splice acceptor site; ψ^+, extended packaging region for high viral titer. *B*, Gp-specific RNA is detected by Northern analysis. Total RNA from NIH 3T3 cells transduced with RV-gp or RV-neo, uninfected NIH 3T3 cells, and LCMV-infected NIH 3T3 cells were hybridized with an LCMV-gp-specific probe. *c*, RNA gel stained with ethidium bromide confirmed that equal amounts of RNA were loaded in each lane.

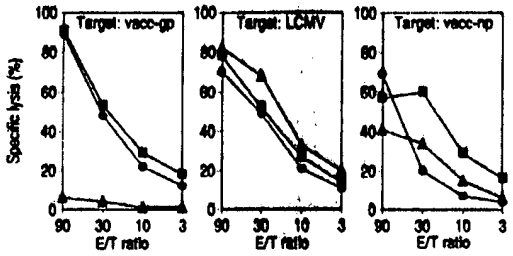

FIGURE 2. LCMV-gp-specific tolerance occurs in chimeric animals reconstituted with bone marrow transduced with RV-gp. Absence of LCMV-gp specific cytotoxic T cells in RV-gp chimeric mice. RIP-gp mice reconstituted with RV-neo (circles) and RV-gp (triangles) transduced bone marrow and control C57BL/6 mice (squares) were infected with LCMV. Spleen cells were assayed for cytotoxic function in a standard chromium release assay with LCMV-infected fibroblasts (MC57G), fibroblasts infected with a vaccinia recombinant virus that expresses LCMV-gp, or a vaccinia recombinant virus that expresses LCMV-np. Less than 5% specific lysis was detected using targets infected with control vaccinia virus alone (data not shown).

peripheral T cell repertoire, but a noteable absence of transgenic $CD8^+$ T cells was observed.

Spleen cells from chimeric animals reconstituted with TCR transgenic bone marrow TCR (RV-gp) and TCR (RV-neo) were also used in a proliferation assay. LCMV-gp-specific proliferative responses remained undetectable in animals reconstituted with TCR (RV-gp), whereas LCMV-specific proliferation was found using T cells from control TCR transgenic mice and control TCR (RV-neo) chimeric mice (Fig. 3*B*). Taken together, these studies demonstrated that LCMV-gp-specific tolerance is induced primarily by clonal deletion in animals reconstituted with RV-gp transduced bone marrow.

To examine whether the retrovirus was detectable in RV-neo and RV-gp chimeric mice, DNA was extracted from various tissues. PCR analysis was done using neomycin-specific primers, and Southern blot analysis of amplified DNA using a neomycin-specific probe demonstrated that the retroviral DNA was present in the bone marrow, thymus, spleen, and lymph nodes from chimeric mice (Fig. 4). Retroviral DNA was consistently detected in primary and secondary lymphoid organs of several RV-gp and RV-neo chimeric mice that had been reconstituted with RV-gp and RV-neo transduced bone marrow for 10 wk to 5 mo.

The most important question, however, was whether retroviral-mediated gene therapy could prevent autoimmunity in vivo. RV-gp and RV-neo transduced donor bone marrow were used to reconstitute transgenic RIP-gp host animals. Chimeric animals and control RIP-gp were infected with LCMV and the glucose levels were monitored. RIP-gp animals became hyperglycemic within 10 days after infection with LCMV as previously reported (5). Similarly, all RIP-gp (RV-neo) chimeric mice (6/6) developed overt hyperglycemia, while none of the RIP-gp (RV-gp) chimeric animals (0/14) developed hyperglycemia (Fig. 5 and Table I). Therefore, using this in vivo system, autoimmunity may be prevented by retroviral-mediated therapy.

Discussion

Several approaches have been taken to control T cell-mediated autoimmune diseases (30). Using animal models, treatment with anti-CD3 (31), anti-CD4, or anti-CD8 mAbs (24, 32, 33) have abrogated disease. The current clinical approach is to control the pathogenic T cells by using immunomodulatory drugs. However, these treatments are accompanied by other side effects as well as general immunosuppression, and therefore an approach that is directed specifically against the pathogenic T cells would be desirable.

Many studies have demonstrated that the induction of T cell tolerance specific for the target self Ag have resulted in the prevention

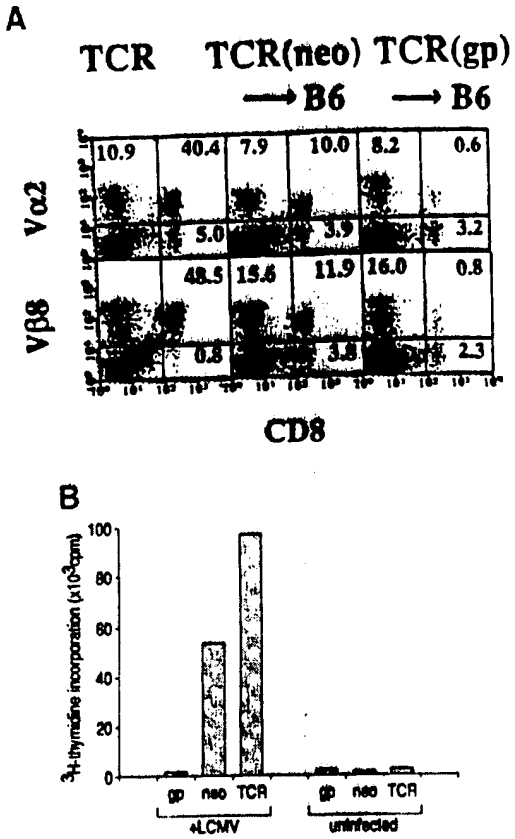

FIGURE 3. *A*, LCMV-gp-specific T cells are absent in the peripheral T cell repertoire. Two-parameter flow cytometry with either mAbs B20.1 (Vα2) or KJ16 (Vβ8.1, 8.2) and CD8 were used to detect transgenic TCR specific for LCMV-gp presented in the context of H-2D^b. Control TCR transgenic mice and chimeras reconstituted with RV-neo or RV-gp transduced TCR transgenic bone marrow are shown. *B*, LCMV-gp-specific proliferation is absent in chimeric mice reconstituted with TCR transgenic bone marrow transduced with RV-gp. Spleen cells from TCR transgenic mice or TCR bone marrow chimeric mice transduced with RV-gp and RV-neo were examined for a primary proliferative response to LCMV by cocultivation with LCMV-infected or uninfected peritoneal macrophages. SD from triplicate samples was <15% of mean. Chimeric animals were generated as described and tested 10 to 12 wk after reconstitution. Data are representative of animals from 4 to 6 independent experiments.

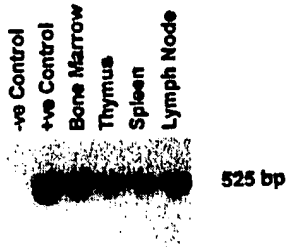

FIGURE 4. Retroviral DNA is detectable in RV-gp chimeric animals. DNA from various tissues was amplified by PCR using neomycin-specific primers, and analyzed by Southern blot with a neomycin-specific probe. Positive control is tail DNA from a gene-deficient mouse that has two copies of the neomycin gene, and negative control is tail DNA from a C57Bl/6 mouse. Similar findings have been shown for several chimeric mice generated using either RV-neo or RV-gp.

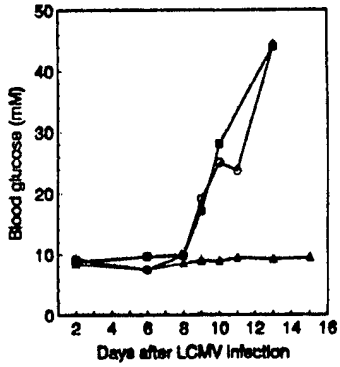

FIGURE 5. Hyperglycemia does not occur in bone marrow chimeric animals expressing RV-gp. RIP-gp transgenic mice were used as controls (squares) or bone marrow chimeras RIP-gp(RV-neo) (circles) or RIP-gp (RV-gp) (triangles) were infected with LCMV. RIP-gp (RV-gp) chimeras were monitored for 40 days, and hyperglycemia was not detected. Chimeric animals were generated as described and assayed after 3 to 5 mo.

Table I. *Autoimmunity does not occur in transgenic mice tolerized with RV-gp*

Mice	*N*	Hyperglycemia[a]	Day of Onset
RIP-gp	6	6	9
RIP-gp (RV-neo)	6	6	9
RIP-gp (RV-gp)	14	0	

[a] All hyperglycemic mice reached nonfasting glucose levels of a minimum of 20 mM.

of disease. Strategies that have been used involve transplanting the affected organ in the thymus to induce central tolerance (34, 35) or tolerizing autoreactive T cells by exposure to high amounts of the target self peptide or autoantigen in neonatal or adult mice (36–41). Vaccination against the autoantigen (42) or the agent that induces autoimmunity (37) have also reduced the incidence of autoimmunity in treated animals.

Other approaches to control autoimmunity have been based on the observation that the disease may be mediated by T cells that use a predominant TCR variable (V) region (4, 43–46). This finding has lead to therapies that control disease by using mAbs specific for the predominant TCR V regions (47), or treatment with peptides specific for a specific V region or autoreactive T cell lines to induce controlling cell populations (39, 48–52). However, these therapies are dependent on the presence of autoreactive T cell populations that utilize a predominant V region, which may not always exist (53–55).

In this study, we report an alternative approach by demonstrating that bone marrow expressing a self Ag through RV transduction can induce specific T cell tolerance and prevent autoimmunity in vivo. Although the target autoantigen must be defined, the advantage of this approach is that the dominant epitopes presented by a given MHC type does not need to be predetermined, and the requirement for preferential V region usage does not exist. Retroviral-mediated gene therapy is attractive because long term T cell tolerance may be induced against a specific autoantigen, thereby leaving an essentially normal functional T cell repertoire in the host. One limitation is that this model is directed towards a single transgenic autoantigen, and the pathogenesis of disease may involve several target autoantigens. Therefore, future studies using this system will examine whether tolerance may be directed towards multiple autoantigens, and will establish modified protocols

to determine the least invasive procedures necessary to induce central or peripheral tolerance.

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Retroviral Gene Transfer into the Intestinal Epithelium

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ABSTRACT

The epithelial cells of the gastrointestinal tract may be attractive targets for somatic gene therapy. In these studies, we have used rats and mice to explore the feasibility of gene transfer into the small intestinal epithelium using retroviral vectors. The first series of experiments was conducted in mature Sprague-Dawley rats using an ecotropic retroviral vector that has bacterial β-galactosidase (β-Gal) as the reporter gene. The vector was introduced into the lumen of ligated segments of terminal ileum. After a 4-hr exposure period, the ligatures were removed. Sham-operated animals were subjected to the same ligation procedure but received only tissue culture medium in the ligated segment. All animals were sacrificed 6 days later, and tissue from both the experimental segment and an upstream control segment was assessed for cytoplasmic β-Gal activity using X-Gal histochemistry. Expression of the reporter gene was observed in the crypt epithelium of tissue exposed to the vector. In the villus epithelium, high background staining precluded accurate assessment of reporter gene expression. To obviate the latter problem, we sought an alternative reporter gene for which there would be no background staining in control animals. We repeated the experiments with β-glucuronidase as the reporter gene in MPS VII mutant mice, which are devoid of this enzyme. In these studies, ileal segments exposed to the vector demonstrated expression of the reporter gene in both the crypt and villus epithelium 4 days after exposure. These results indicate that genes can be transferred into the intestinal epithelium using retroviral vectors introduced luminally. These studies constitute an encouraging first step in the assessment of the intestinal epithelium as a site for somatic gene therapy.

OVERVIEW SUMMARY

There are a number of congenital disorders of the intestinal epithelium that could be amenable to gene therapy (*e.g.*, cystic fibrosis, transporter deficiencies, *etc.*). In addition, the intestine could be used as an alternative site to correct metabolic disorders, such as phenylketonuria, and secretory disorders, such as hemophilias. This paper presents *in vivo* studies in both a rat model and a mouse model using retroviral vectors delivered into the intestinal lumen. The results show successful gene transfer into epithelial cells and thus pave the way for future experiments designed to improve the efficiency of this process.

INTRODUCTION

THE INTESTINAL EPITHELIUM is a continuously renewing monolayer that occupies the interface between the internal and external milieu. Its role in digestion and absorption is facilitated by its extremely large surface area, which results primarily from the presence of villi. Villus epithelial cells are replaced every 2–3 days with cells emerging from the crypts of Lieberkühn. Each villus is fed by 10 or more crypts (Komuro and Hashimoto, 1990), each of which has a zone of stem cells toward its base (Cheng and Leblond, 1974). Kinetic analysis following labeling with [^{3}H]thymidine has led to the prediction that there are between 4 and 16 stem cells per crypt (Potten and

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Loeffler, 1987). Daughters of the crypt stem cells enter a transit zone where they divide approximately four times before leaving the proliferative cycle (Potten and Loeffler, 1987). They then move onto the villi where they acquire various differentiated functions (Gordon, 1989). The large surface area of the epithelium, together with its ease of access *via* the intestinal lumen, makes it an attractive potential site for somatic gene therapy.

Gene transfer into the intestinal epithelium would have two general applications in the field of gene therapy: (i) the correction of genetic and acquired disorders that affect the epithelium, and (ii) as an alternative site of expression of proteins normally expressed in some other tissue. The intestinal derangements associated with cystic fibrosis (CF) would be a good example for the first application. Three of the four published mouse models for CF (Colledge *et al.*, 1992; Dorin *et al.*, 1992; Snouwaert *et al.*, 1992; O'Neal *et al.*, 1993) have severe pathology of the intestine that leads to intestinal obstruction followed shortly by death. Although other genetic disorders of the intestinal epithelium are relatively rare, the epithelium may be used as an ectopic site for correction of various metabolic disorders and deficiencies of secreted proteins. Inasmuch as liver and intestine share numerous metabolic pathways, Jones *et al.* (1990) have suggested that disorders such as deficiencies in the urea cycle or in phenylketonuria may be amenable to intestinal gene therapy. Likewise, the fact that the intestinal epithelium is capable of secreting a foreign protein into the circulatory system (Sweetser *et al.*, 1988) suggests its use for production of proteins such as specifically engineered antibodies, clotting factors, antiproteases, and protein hormones.

The advantages of the intestine as a site for somatic gene therapy are several fold. First is its ease of access *via* the lumenal route which, in humans, would allow direct *in vivo* delivery of vectors using standard endoscopic procedures. A second advantage is that the stem cells, which are the logical targets for gene therapy, are continuously proliferating, thus allowing the deployment of retroviral vectors that are already approved for human investigation. Given that some applications of somatic gene therapy, such as correction of metabolic disorders, may require substantial numbers of cells to be expressing a critical enzyme, a third advantage of the intestinal epithelium is its large mass.

The goal of the present studies was to determine whether gene transfer into the intestinal epithelium could occur *via* luminal delivery of replication-incompetent retroviral vectors in rodent models. Although human application would utilize endoscopic procedures, the lack of availability of such instruments for small rodents necessitated a surgical approach for these exploratory studies. The vectors were introduced into ligated segments of ileum for a 4-hr exposure period. Animals were killed 4–7 days later and assayed for expression of the reporter gene. The results indicate successful gene transfer in both rats and mice, albeit at relatively low efficiency.

MATERIALS AND METHODS

Animals and chemicals

Adult Sprague-Dawley (Crl:CD[SD]BR) male rats weighing 150–175 grams were obtained from Charles River Laboratories (Portage, MI). Male MPS VII mice (gus^{mps}/gus^{mps}) from the B6.C-H-2^{bml}/ByBir-gus^{mps}/ + mutant strain aged 1–5 months (13–29 grams) from The Jackson Laboratory (Bar Harbor, ME) were used. The animals were maintained at 21°C + 1°C with food and water *ad libitum*. All histochemical substrates were from Sigma Chemical (St. Louis, MO).

Retroviral vectors

$Zen^{+}\beta$-Gal (courtesy of Dr. Philippe Soriano) is a recombinant derivative of Moloney murine leukemia virus with the bacterial β-galactosidase (β-Gal) gene driven by the viral long terminal repeat (LTR). It was propagated in the ecotropic packaging cell line $GP^{+}E86$ (Markowitz *et al.*, 1988) and routinely yielded titers in the order of $2–3 \times 10^5$ cfu/ml. The NTK-βGEO construct has the rat β-glucuronidase gene driven by the herpes simplex thymidine kinase promoter (Wolfe *et al.*, 1990). It was propagated in a clone of GP + E86 and concentrated using the Centricell filtration method to yield titers of 10^7 cfu/ml. In all experiments, Polybrene (8 μg/ml) was added to the vector solutions immediately prior to use.

Surgical protocols

Both rats and mice were subjected to the same surgical procedure. Following isoflurane anesthesia (Anaquest, Madison, WI), an incision was made in the lower abdomen. The first Peyer's patch proximal to the ileocecal junction was identified, and an ileal segment (1.5–3 cm) immediately rostral was gently cleared of its chyme by flushing with phosphate-buffered saline (PBS). The segment was ligated with a coarse thread at both ends, and an appropriate volume (1.5 ml for rats, 0.5 ml for mice) of the respective retroviral vector (experimental) or culture medium (sham) was then introduced *via* a 25-gauge needle. With these volumes, the segments were distended sufficiently to expose the intestinal crypts (Sandberg *et al.*, 1994). The animals were allowed to recover from this initial surgery, then 4 hr later they were reanesthetized and the coarse thread ligatures were removed. Fine silk ligatures were loosely anchored at both ends of the experimental segment for recognition at the time of autopsy.

The rats tolerated the procedure very well and were in good health and gaining weight when sacrificed 6 days post-surgery. In contrast, the MPS VII mice displayed high mortality. With only 9 out of the 29 operated mice surviving on the fourth day post-surgery, these animals were sacrificed at this time rather than waiting for the 6-day period used for the rats. The MPS VII mice have a high mortality rate from their severe degenerative disease (Vogler *et al.*, 1990; Birkenmeier *et al.*, 1991). Of the 9 surviving mice, 4 were sham-treated and 5 were vector-treated.

Histochemical detections

For both rats and mice, at the time of sacrifice the experimental segment (as defined by the marker ligatures) was removed together with a control segment taken 10 cm and 6 cm upstream, respectively. After flushing with 0.9% NaCl to remove luminal contents, the segments were frozen in OCT (Miles, Elkhart, IN) for subsequent cryostat sectioning and histochemical analyses. Frozen sections (8–10 μm) were made

transverse to the longitudinal axis of the ileum. Consecutive sections were collected at varying intervals along the length of the respective segments t be analyzed. In the rat tissues, β-Gal was detected histochemically using the chromagen X-Gal as a substrate under standard conditions. A section of transgenic mouse liver expressing bacterial β-Gal (courtesy of Dr. Savio Woo) was run with each set as a positive control. In the MPS VII mouse tissues, β-glucuronidase was detected using naphtol-AS-BI β-D-glucuronide and pararosaniline hydrochloride as described by Birkenmeier *et al.* (1989). A section of nonmutant liver expressing β-glucuronidase was run as a positive control. In both rat and mouse studies, the sections were scored blindly, and positive epithelial cells in the villus and crypt regions were counted in both upstream (control) and experimental segments.

RESULTS

β-*Gal expression in rats*

Because endogenous lactase present in the apical membrane of villus epithelial cells is also detected by X-Gal histochemistry, observers scored as positive cells only those which contained cytoplasmic staining (the expected location of the bacterial β-Gal reporter gene). The number of rats exhibiting positive β-Gal scores from this procedure is shown in Table 1A. It can be seen that in the experimental segment, all animals displayed positive cells in the crypt epithelium, and 2 out of 3 animals had positive villus epithelial cells. None of the animals had positive cells in the crypts of the upstream control segments. However, there was a high incidence of animals having positive scores in the villi of control tissue.

The number of β-Gal-positive cells observed in the experimental and control segments of the above rats are presented in Table 1B. In the crypts, where background staining was not seen in control tissue, experimental segments displayed 2–11 positive epithelial cells in the four sections examined. Figure 1 shows an example of the histology. Although sectioning was performed transverse to the long axis of the intestine, crypts and villi were cut transversely at times. The upstream control section in Fig. 1A shows crypts in complete cross section, whereas the experimental section (Fig. 1B) is somewhat at an angle. Note that control tissue shows no blue reaction product whereas the experimental section (*i.e.*, the ligated region that was exposed to the vector) has several cells with a distinct positive reaction in the cytoplasm (see arrow). Because of the angle of the experimental section, positive cells were seen at three different levels of the crypts: The three crypts in the uppermost part of the picture (solid vertical arrows) are sectioned somewhere near their middle, the crypt in the center of the picture (open arrow) is sectioned close to the base (thus, the more dense appearance of the epithelial cells), and the two lowest crypts (horizontal arrow) are sectioned right at the base (thus showing no lumen).

In contrast to the clear results in the crypts, background β-Gal staining on the villi was a serious problem. As can be seen in Table 1B, the number of positive villus cells was just as high in upstream control tissue as in experimental tissue. Sham-operated animals (data not shown) exposed to culture medium instead of the retroviral vector showed a similar number of positive cells as in the villi of upstream segments. Because of this high incidence of endogenous β-Gal in villus epithelium, an alternative animal model/reporter gene system was sought.

TABLE 1. EXPRESSION OF CYTOPLASMIC β-GAL IN RAT TISSUES

			Crypt epithelium	*Villus epithelium*
A. Proportion of animals positive[a]				
Experimental segment			3/3	2/3
Control segment			0/3	3/3
		Number of sections examined	*Total number of positive cells*	*Total number of positive cells*
B. Number of positive cells				
Experimental segment:	Rat #2	4	2	2
	Rat #4	4	5	2
	Rat #9	4	11	0
Control segment:	Rat #2	4	0	2
	Rat #4	4	0	1
	Rat #9	2	0	3

[a]Data are shown as the number of animals with one or more positive scores in any of the sections taken from either the experimental segment or the upstream control segment.

β-*Glucuronidase expression in MPS VII mice*

Table 2A shows that experimental segments of 2 out of 5 mice exposed to the β-glucuronidase vector showed positive epithelial cells in both villus and crypt regions. There were no positive cells in the upstream control tissue of vector-treated mice or in any segments from sham-treated animals. Table 2B shows the number of positive cells observed in both crypt and villus epithelium. It can be seen that the incidence of positive epithelial cells is substantially higher on the villi than in the crypts. Of interest is the fact that in one of these mice (#24), positive villus cells were all localized on two to three adjacent villi. Examples of histology from mouse #24 are shown in Fig. 2. Figure 2A shows that upstream control tissue was devoid of the pink reaction product of the β-glucuronidase assay. In contrast, the tissue from the experimental segment displayed substantial pink staining that could be followed through 15 adjacent sections. Figure 2B, C, and D show examples at different intervals throughout this region. These sections being 8 μm thick, it can be concluded that cells from that region were expressing the β-glucuronidase gene over at least a 120-μm-long segment. None of the sham-operated mice exposed to the culture medium instead of the retroviral vector showed any positive cells.

DISCUSSION

The experiments described in this paper represent an *in vivo* attempt to explore the feasibility of using the intestinal epithe-

lium as a site for somatic gene therapy. Given the continual proliferation f this tissue, retroviral vectors were the most appropriate choice because retroviruses are known to infect dividing cells and to integrate their genome into the host DNA. Moreover, we had previously established that the mRNA for the ecotropic retroviral receptor is expressed in the intestinal mucosa and appears to be more abundant in proliferating epithelial cells than in differentiated cells (Puppi and Henning, 1995). We had also conducted studies with IEC-6 cells, a rat small intestinal crypt cell line, and found that these cells are readily transduceable by ecotropic retroviral vectors (Noel *et al.*, 1994).

The initial animal model we chose to explore was the Sprague-Dawley rat. Given the complex morphology of the intestinal mucosa, the use of a reporter gene that could be detected histochemically was preferred. Thus, in the rat experiments, we used the Zen$^+$β-gal retroviral vector, which has bacterial β-Gal as the reporter gene. All slides were scored blindly by two independent observers. Using this approach, appreciable numbers of false positives were found on the villi in both upstream control tissue and tissue from sham-operated animals. Because the latter animals had not been exposed to the retroviral vector, we conclude that this background staining represents endogenous β-Gal activity, most probably lactase *en route* to the apical membrane. The fact that these background problems were confined to villus epithelial cells (as compared with crypt epithelial cells) supports this conclusion, because lactase is known to be expressed specifically on the villus (Dudley *et al.*,1992; Duluc *et al.*, 1993). Although no conclusions regarding expression of the reporter gene can be drawn from the villus data, the crypt data are not compromised. In crypt epithelial cells, the consistent finding of β-Gal-positive cells in tissue exposed to the retroviral vector suggests that successful gene transfer had indeed occurred.

To obviate the villus background problems in our rat/β-Gal studies, we sought an alternative model in which the reporter gene could still be detected histochemically but in which the background in nonexposed segments would be zero. The use of β-glucuronidase as a reporter gene in MPS VII mice appeared to fulfill both of these criteria. In agreement with earlier studies of other tissues (Birkenmeier *et al.*, 1991), we found the intestinal epithelium of adult MPS VII mice to be lacking β-glucuronidase as assessed by histochemistry. Just as with our β-Gal studies, all slides were scored in a blinded fashion. Both sham-operated and upstream control tissue showed no β-glucuronidase staining. In contrast, experimental segments showed clear evidence of reporter gene expression in both the crypt and villus epithelium. Although luminal contents (*e.g.*, nutrients, bacteria, milk) might contain β-glucuronidase activity, the present data, in which a total of 1,098 sections of control tissue (upstream and sham-treated) yielded no positive intestinal epithelial cells, indicate that the positive cells in the treated animals were expressing β-glucuronidase activity from the vector.

Within the intestinal crypts, there are two potential populations of target cells for retroviral vectors, namely the stem cells and the transit population of dividing cells. Given that cells take approximately 36 hr to pass through the transit zone (Potten and Loeffler, 1987), the persistence of the reporter gene in the crypts 6 days and 4 days following gene transfer in the rats and mice, respectively, suggests that stem cells were transduced. However, definitive proof for this will require studies at longer time periods.

We conclude from both the rat and mouse studies that retroviral transduction of the intestinal epithelium is possible. With existing techniques, however, the efficiency of transduction is very low. For example, our best rat showed an average of approximately three positive crypt cells per intestinal cross section. Given that these sections contained an average of 40 crypts and that each crypt averaged 50 cells in length, there were approximately 2,000 crypt cells per section. Thus, the incidence of positive crypt cells in the rats was in the order of 3/2,000 or 0.15%. Similar calculations for our best mouse gives approximately the same incidence. Moreover, in the mouse studies, only 40% of experimental mice displayed transduction. Similar degrees of variability have been observed in other tissues fol-

TABLE 2A. NUMBER OF MPS VII MICE EXPRESSING β-GLUCURONIDASE IN INTESTINAL EPITHELIUM

Group	*Segment*	*Crypt*[a]	*Villus*[a]
Vector-treated	Experimental	2/5	2/5
	control	0/5	0/5
Sham-treated	Experimental	0/4	0/4
	control	0/4	0/4

[a]Data shown as the number of animals with positive crypt or villus epithelial cells compared with the total number of animals in that group.

TABLE 2B. NUMBER OF β-GLUCURONIDASE-POSITIVE CELLS OBSERVED IN INTESTINAL EPITHELIUM OF MPS VII MICE

Identification of tissues	*Mouse*	*Number of sections examined*	*Total number of positive cells*	
			Crypt	*Villus*
A. Vector-treated mice[b]				
Experimental segment:	#15	47	2	9
	#24	72	5	80
Control segment:		486	0	0
B. Sham-treated mice[b]				
Experimental segment:		293	0	0
Control segment:		319	0	0

[b]Data for positive tissue are shown for individual mice; those for control tissue are shown collectively.

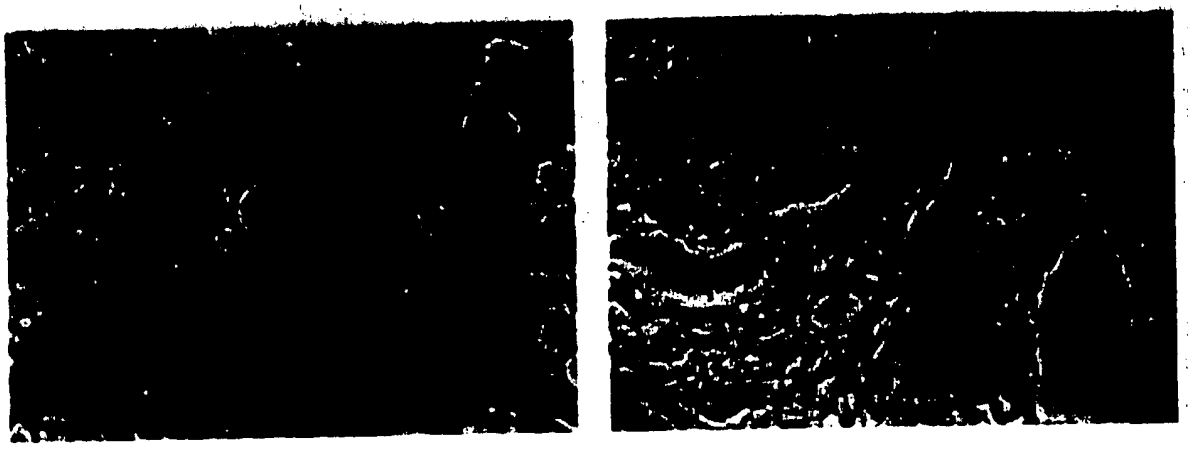

FIG. 1. Histochemical assays for β-Gal in crypt region of upstream control tissue (A) and experimental tissue (B). Experimental tissue had been exposed to the Zen$^+$ β-gal retroviral vector 6 days prior to sacrifice. The two sections shown were taken from the same animal. Magnification, 400×.

lowing exposure to retroviral vectors (Hatzoglou *et al.*, 1990; Clapp *et al.*, 1991; Kay *et al.*, 1992). Generic causes of such variability include lability of the vectors and the relatively small proportion of the cell cycle during which exposure must occur (Miller *et al.*, 1990). In our experiments, other contributors to the variability and low efficiency of gene transfer could have been inadequate numbers of vector particles and poor penetration of intestinal crypts. Although experimental segments were distended to open the crypts as described by Harris *et al.* (1988), in these experiments there was no attempt to remove mucus. Subsequent studies in our laboratory have established conditions for *in vivo* mucus removal (Sandberg *et al.*, 1994) that should be employed in future studies of gene transfer into this tissue.

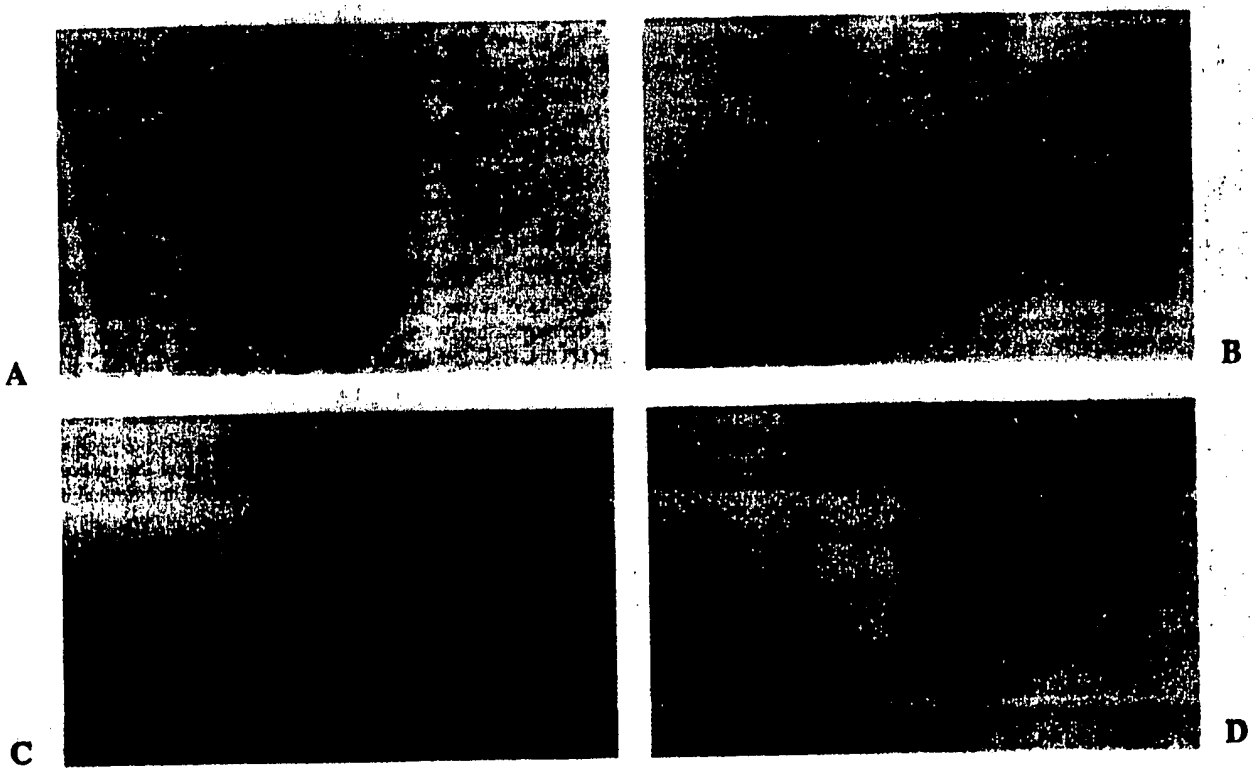

FIG. 2. Histochemical assay for β-glucuronidase in upstream control (A) and experimental (B, C, D) tissues from an MPS VII mouse. Experimental tissue was exposed to the NTK-βGEO retroviral vector 4 days before sacrifice. The sections shown were taken from the same animal (#24). Magnification, (A) 100×; (B, C, D) 200×.

In summary, we have demonstrated the feasibility of *in vivo* gene transfer into the intestinal epithelium using retroviral vectors. Clearly, there are problems to be overcome to achieve high-efficiency gene transfer as well as to decrease the variability between animals. Nevertheless, the potential advantages of the intestine as a site of somatic gene therapy indicate that further studies with this system should constitute a promising avenue of future research. With a view to ultimate clinical application, it is important to keep in mind that, although our studies with rodents used a surgical approach, in the human, luminal delivery of vectors to both the small and large intestine is conceivable *via* upper and lower endoscopic procedures, respectively. This is an approach that we are presently investigating in the large intestine of rats. Even if efficiencies of transduction remain relatively low, the ease of endoscopic delivery would allow multiple deliveries of vector to be performed, if necessary. Likewise, if the stem cell population proves difficult to transduce, repeated deliveries aimed at the transit cell population may also have therapeutic value.

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